Correcting miR-15a/16 genetic defect in New Zealand Black mouse model of CLL enhances drug sensitivity

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Abstract

Alterations in the human 13q14 genomic region containing microRNAs mir-15a and mir-16-1 are present in most human chronic lymphocytic leukemia (CLL). We have previously found the development of CLL in the New Zealand Black murine model to be associated with a point mutation in the primary mir-15a/16-1 region, which correlated with a decrease in mature miR-16 and miR-15a levels. In this study, addition of exogenous miR-15a and miR-16 led to an accumulation of cells in G1 in non–New Zealand Black B cell and New Zealand Black–derived malignant B-1 cell lines. However, the New Zealand Black line had significantly greater G1 accumulation, suggesting a restoration of cell cycle control upon exogenous miR-15a/16 addition. Our experiments showed a reduction in protein levels of cyclin D1, a miR-15a/16 target and cell cycle regulator of G1/S transition, in the New Zealand Black cell line following miR-15a/16 addition. These microRNAs were shown to directly target the cyclin D1 3′ untranslated region using a green fluorescent protein lentiviral expression system. miR-16 was also shown to augment apoptosis induction by nutlin, a mouse double minute 2 (MDM2) antagonist, and genistein, a tyrosine kinase inhibitor, when added to a B-1 cell line derived from multiple in vivo passages of malignant B-1 cells from New Zealand Black mice with CLL. miR-16 synergized with nutlin and genistein to induce apoptosis. Our data support a role for the mir-15a/16-1 cluster in cell cycle regulation and suggest that these mature microRNAs in both the New Zealand Black model and human CLL may be targets for therapeutic efficacy in this disease. [Mol Cancer Ther 2009;8(9):2684–92]

Introduction

MicroRNAs are small, evolutionarily conserved, noncoding single-stranded RNAs that regulate gene expression by binding with a RNA-induced silencing complex (RISC)-like complex to the 3′ untranslated region (UTR) of target mRNA (1, 2). Loss or amplification of microRNAs are reported in various cancers, affecting cell cycle progression or survival mechanisms (3) by acting as either tumor suppressors or oncogenes (4).

Chronic lymphocytic leukemia (CLL), a malignancy of the CD5+ B cell, is the most common leukemia to affect adults in the Western world (5). More than 50% of CLL cases exhibit a deletion within the 13q14 chromosomal region (6) containing the DLEU2 gene (7, 8). DLEU2 contains microRNAs mir-15a and mir-16-1, which are also deleted or down-regulated in a subpopulation of patients with B-cell CLL (9, 10). The New Zealand Black mouse, a de novo model for autoimmunity (11) and CLL (12, 13), exhibits a T→A point mutation six bases downstream from pre-mir-16-1 on chromosome 14 (14), similar to the C→T point mutation seen in human CLL (15), which may affect structural stability of the stem loop and proper processing, resulting in decreased miR-15a and miR-16 expression.

Similar to CLL, the disease in New Zealand Black mice is an age-associated malignant expansion of poly–reactive CD5+, B-1 clones (13, 16). Most B-1 clones are IgM+ B220 (CD45R)dull, and CD5null, and often possess chromosomal abnormalities (17). The germline genetic alterations in the mir-16-1 locus in New Zealand Black mice are correlated with a decrease in mature miR-16 expression in lymphoid tissues (14). The New Zealand Black also displays mild autoimmune associated with B-cell hyperactivity, resulting in autoimmune hemolytic anemia and antimicrobial antibodies (13), similar to autoimmunity seen in some CLL patients, resulting from the production of autoantibodies (18). CLL was classically defined as a progressive accumulation of resting B cells possessing a defect in the apoptotic pathway (19). Although leukemic cells circulating in the peripheral blood have been shown to be arrested in G_0/G_1 (20), CLL is now thought to be more dynamic, with a detectable level of cell division occurring at all times (21). In lymphoid tissue, leukemic cells appear as aggregates termed “pseudofollicles” or “growth centers,” in which neighboring CD4+ CD40L+ T cells induce their proliferation (20).
MicroRNAs have been found to have an impact on cell cycle progression (22–25). MicroRNAs, such as miR-106b, miR-221, and miR-222, have been found to promote cell cycle progression in various human solid tumors (22, 23), and miR-16 and let-7b have been reported to contribute to G1 accumulation in human colon carcinoma cell lines and melanoma cells, respectively (24, 26).

In this study, we sought to define a role for miR-16 in the malignant B-1 cell growth and persistent nature seen in the New Zealand Black model of CLL by examining the effects of exogenous miR-16 on our New Zealand Black–derived malignant B-1 cell line (LNC) that mimics the late stages of CLL (27). We were interested in the effects of miR-16 on its targeted cell cycle components, particularly cyclin D1. Although overexpression of cyclin D1 among leukemias and lymphomas is a hallmark of mantle cell lymphoma, resulting from translocation t(11;14)(q13;q32) (28, 29), increased expression of the New Zealand Black derived malignant B-1 cell line. The results of our study suggest that aberrant expression of miR-16 plays a role in deregulated cell cycle transit and drug sensitivity, and upon restoration of miR-16 levels to malignant B-1 cells, uncontrolled proliferation can be impeded and selective killing of malignant B-1 cells can be enhanced.

Materials and Methods

Sources of Cells

Mice, New Zealand Black/BLNJ and C57Bl/6, were obtained from Jackson Laboratories. The New Zealand Black–derived malignant B-1 cell line, LNC, was derived from a year-old New Zealand Black, as previously described (27). The BALB/c derived B-cell line, A20, was obtained from American Type Culture Collection. The NFS-1.0 B-cell line was derived from the NFS/N strain (36) and a kind gift from Dr. Steven Bauer, Center for Biologics Evaluation and Research, Food and Drug Administration. A20 and NFS-1.0 were used as non–New Zealand Black B-cell lines (both having normal levels of miR-15a and miR-16), and C57Bl/6 mice were used as non–New Zealand Black mice, having normal levels of miR-15a and miR-16.

Cell Sorting of Spleen Cells

Spleens were removed from 9- to 14-mo-old C57Bl/6 (control non–New Zealand Black strain) and New Zealand Black mice; made into single-cell suspensions that were four-color stained with antibodies directed against IgM fluorescein isothiocyanate, B220 PerCP-Cy5.5, CD4 phycoerythrin, and CD25 allophycocyanin (Invitrogen); and sorted into four distinct populations on a FACS Aria (Becton Dickinson). The B cells were gated on CD4+ and sorted into two populations (B-2 cells, CD4+/IgM+/CD25bright; B-1 cells, CD4+/IgM+/CD25dim). T cells were sorted into two populations (IgM+/CD4+/CD25− and Tregs, IgM+CD4+/CD25−; not shown).

Collection of Synchronized Cell Fractions

Centrifugal elutriation was done on New Zealand Black and non–New Zealand Black B-cell lines harvested at 4 to 8 × 10^5 cells/mL using the Beckman Coulter J6-M1, set at 2,700 rpm and 18°C. Cell fractions G0/G1 (G1), late G1/early S (G1/S), S, late S/G2 + M (S/G2) were collected at flow speeds of 27, 30, 37, and 45 mL/min, as previously described (37). EDTA (1 mmol/L) in 1× PBS was used for the New Zealand Black cell line elutriation buffer, and 5 mmol/L EDTA in 1× PBS was used for the non–New Zealand Black cell line.

Transfection of Cells with miR-15a and miR-16 Mimics

Asynchronous populations and elutriated fractions G1, G1/S, S, and S/G2 were transfected using the Amaxa Nucleofector II instrument (Lonza Walkersville, Inc.) using programs L-013 (for BALB/c line), X-001 (for NFS/N line), and G-016 (for New Zealand Black line) with solutions V (for normal B lines) and T (for New Zealand Black cell line). Cells were transfected with 3 μg miRIDIAN Mimic mmu-miR-15a, miRIDIAN Mimic mmu-miR-16, or miRIDIAN Mimic Negative Control 1 (a microRNA mimic-like duplex with a nonspecific sequence; Dharmacon), according to the manufacturer’s protocol. Samples were plated at 5 to 6 × 10^5 cells/mL and placed in a 37°C CO2 incubator.

Cell Cycle Analysis

Cells (untreated, transfected, genistein treated) were stained with hypotonic propidium iodide (0.05 mg/mL propidium iodide, 0.1% Triton X-100, 0.1% sodium citrate) at either 24 or 48 h posttreatment, acquired on Becton Dickinson FACScalibur using CELLQuest software (Becton Dickinson), and analyzed using ModFit LT V3.1 software (Verity Software House).

MicroRNA Extraction and Quantification

Total RNA, including microRNA, was extracted from transfected and untransfected cells, according to the Trizol (Invitrogen) manufacturer’s protocol. Quantitative real-time PCR was used to quantify mature miR-16 expression from tissues and transfected samples using the TaqMan microRNA Reverse Transcription and TaqMan microRNA hsa-miR-16 Assay Kit (Applied Biosystems). The reverse transcription reaction was done using the GeneAmp PCR System 9600 (Perkin Elmer), and quantitative real-time PCR was run on the Applied Biosystems 7500 Real-Time PCR Systems for 40 cycles at 60°C. A standard curve was generated using serial femtomole dilutions of miR DIAN Mimic miR-16 (Dharmacon) to quantitate amount of miR-16 present before and posttransfection with miR-16. Real-time PCR was done to evaluate the expression of miR-16 in spleen cells isolated from New Zealand Black and normal mice using the TaqMan microRNA hsa-miR-16 Assay Kit, according to the manufacturer’s protocol. The relative quantification values of New Zealand Black miR-16 levels compared with

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normal mouse strain miR-16 levels were determined using
the standard 2−ΔΔCT method, according to the manufacturer's protocol. The total amount of input RNA was normalized to either Taqman 18S rRNA or U6 small nuclear RNA (Applied Biosystems).

Analysis of Cyclin D1 mRNA Levels

Total RNA was extracted from samples 24 h posttransfection with negative control or miR-16 mimic, as described above, and subjected to endpoint PCR to detect cyclin D1 mRNA levels. The reverse transcription step was executed using random hexamers and run on GeneAmp PCR System 9600 (Perkin Elmer). The cyclin D1 647-bp message was amplified on the GeneAmp 9600 at 60°C for 35 cycles using primers 5′-GCACACCTCTGGCTCTGTC-3′ (forward) and 5′-GCACCAGAGACTCAGAGC-3′ (reverse), normalizing the amount of input RNA to hypoxanthine-guanine phosphoribosyltransferase. The following primers were used to distinguish cyclin D1a isoform from cyclin D1b (data not shown): 5′-CAGACATCGCAAGCATGC-3′ (forward primer for D1a and D1b), 5′-CGACGTGGAGATGTG-3′ (reverse D1a), and 5′-GCATCAGGAGCTGTGCACT-3′ (reverse D1b). PCR products were then run on a 2% agarose gel and quantified using the Typhoon 9410 Variable Mode Imager (Amersham Biosciences) and ImageQuant 5.2 densitometric analysis software (Molecular Dynamics).

Analysis of Cyclin D1 Protein Levels

Predicted targets of miR-15a and miR-16, including cyclin D1, were obtained from TargetScan Whitehead Institute for Biomedical Research. The protein levels of target cyclin D1 were determined in transfected and untransfected cells (24 h posttransfection with miR-15a, miR-16, or negative control) by flow cytometric analysis. Cells were fixed in 2% paraformaldehyde and permeabilized with Triton X-100 (Sigma). Permeabilized cells were then stained with fluorescein isothiocyanate isothiocyanate–conjugated anti-cyclin D1 (Biosource, Invitrogen). Single-color histogram samples were acquired on FACSCalibur (Becton Dickinson), collecting 2 × 10^4 events, and analyzed using CELLQUEST Software (Becton Dickinson).

Construction of Lentiviral Vector

A 647-bp fragment of the cyclin D1 3′ UTR (chromosome 7:3185751-3186397) containing three predicted miR-16 target sites according to TargetScan was cloned using the following primers: forward, 5′-CAGACATCGCAAGCATGC-3′; reverse D1b (5′-GCATCAGGAGCTGTGCACT-3′). PCR products were then run on a 2% agarose gel and quantified using the Typhoon 9410 Variable Mode Imager (Amersham Biosciences) and ImageQuant 5.2 densitometric analysis software (Molecular Dynamics).

Lentivirus Sensor Assay

Our New Zealand Black malignant B-1 cell line was transduced separately with the Bd.LV.GFP vector lacking the cyclin D1 3′ UTR sequence and with Bd.LV.GFP vector containing the cyclin D1 3′ UTR sequence (because these are lentiviral vectors, they stably integrate into the genome of the cells). The transduced lines were then transfected with 3 μg miR-15a, miR-16, or negative control (Dharmacon). Cells were incubated at 37°C, and flow cytometric analysis for GFP and monomeric Cherry (mCherry) expression was performed at 24 h. One hundred thousand events per sample were acquired on the LSR II (BD Biosciences) and analyzed using FACS Diva Software (BD Biosciences). Five thousand events per sample were acquired on the Amnis ImageStream IS100

Figure 1. miR-16 expression is decreased in New Zealand Black B cells. A, spleen cells from 10 to 14 mo New Zealand Black and non–New Zealand Black strain mice were stained and sorted (three mice for each strain in three independent sorts). A representative sort from an individual New Zealand Black spleen. The initial population of CD44− cells stained for IgM and B220 (CD45R; left) were sorted into conventional B-2 (IgM+, B220−; right top) and B-1 cells (IgM+, B220−; right bottom). B, real-time PCR was done on RNA extracted from each population. The relative quantification of miR-16 levels in New Zealand Black mice compared with non–New Zealand Black strain was determined using the standard 2−ΔΔCT method, miR-16 expression analysis in each population is reported relative to the expression in the non–New Zealand Black strain mouse B-2 population (normal strain B-2 = 1). (black columns, B-2; white columns, B-1 values). Error bars, the SEM for three independent sorts. *, statistical significance between miR-16 levels of New Zealand Black B-1 and B-2 cells compared with C57 B-1 cells (P < 0.05).
appropriate to determine statistical significance, $P$ to calculate the SEM. Student’s $t$ test was used whereby $P < 0.02$.

**Results**

**miR-16 Expression Is Decreased in Subpopulations of New Zealand Black B cells**

Because the New Zealand Black spleen has decreased levels of miR-15a (data not shown) and miR-16 (14) as compared with non–New Zealand Black strain spleens, we wanted to further determine whether New Zealand Black malignant B-1 cells had a selective decrease in miR-16 expression. We analyzed sorted subpopulations containing nonmalignant B cells and malignant B cells. Spleen cells obtained from New Zealand Black and non–New Zealand Black mouse spleens (10–12 months of age) were sorted into conventional B-2 cells (CD4$^+$, IgM$^+$, B220$^+$) or B-1 cells (CD4$^-$, IgM$^+$, B220$^{null}$; Fig. 1A). RNA was obtained from the sorted B-cell populations, and the level of miR-16 expression was determined through real-time PCR. As anticipated, New Zealand Black conventional B-2 cells and B-1 cells (containing the malignant B clone) had less than half the amount of miR-16 expression relative to the B-2 and B-1 cell populations from age-matched non–New Zealand Black mouse spleens (Fig. 1B). This indicates that all B cells from New Zealand Black mice are affected by the point mutation in the $mir$-$15a$/$16$-1 loci, resulting in decreased miR-16 expression.

**Restoration of miR-16 Levels in G1 Arrest in New Zealand Black–Derived Malignant B-1 Cell Line**

In the New Zealand Black spleen, most B-2 cells are in $G_1$, whereas the malignant B-1 cells are cycling (14). To determine the effects of miR-16 on growth and cell cycle transit of these malignant B-1 cells, New Zealand Black B-1 and non–New Zealand Black B-cell lines were transfected with 3 μg of miR-15a or miR-16 mimic or a negative control mimic (because miR-16 shares a seed sequence and many predicted targets with miR-15a, we also transfected cells with a miR-15a mimic to test if both microRNAs would have the same effect on the cells). To observe cell cycle progression following transfection, cells were stained with hypotonic propidium iodide at 24 hours posttransfection and analyzed using ModFit LT V3.1 Software. New Zealand Black and non–New Zealand Black B-cell lines exhibited an increase in $G_1$ following transfection with miR-15a and miR-16 (similar to previous reports by our group and others; refs. 14, 24), whereas the malignant B-1 cells are cycling (14). To determine the effects of miR-16 on growth and cell cycle transit of these malignant B-1 cells, New Zealand Black B-1 and non–New Zealand Black B-cell lines were transfected with 3 μg of miR-15a or miR-16 mimic or a negative control mimic (because miR-16 shares a seed sequence and many predicted targets with miR-15a, we also transfected cells with a miR-15a mimic to test if both microRNAs would have the same effect on the cells). To observe cell cycle progression following transfection, cells were stained with hypotonic propidium iodide at 24 hours posttransfection and analyzed using ModFit LT V3.1 Software. New Zealand Black and non–New Zealand Black B-cell lines exhibited an increase in $G_1$ following transfection with miR-15a and miR-16 (similar to previous reports by our group and others; refs. 14, 24), yet $G_1$ accumulation in the New Zealand Black B-cell line was significantly higher than that in the non–New Zealand Black B-cell line (Fig. 2). The New Zealand Black cell line also exhibited a significant decrease in $S$ phase as compared with the non–New Zealand Black B-cell line (Fig. 2).

The New Zealand Black B-cell line was examined for any selective decrease in miR-16 specific to a cell cycle phase. B-cell lines were fractionated by cell cycle phase using centrifugal elutriation into the following fractions: fraction 1, early to mid-$G_1$ ($G_1$); fraction 2, late $G_1$/early $S$ ($G_1$/S); fraction 3 ($S$); and fraction 4, late $S$ to $G_2$/M ($S$/$G_2$). Centrifugal elutriation separates cells in a particular cell cycle phase on the basis of size, avoiding the artifacts associated with other cell synchronization techniques, such as altered gene expression (41). Representative results obtained following separation are shown (Fig. 3A). Analysis of basal levels of mature miR-16 expression revealed that the New Zealand Black–derived B-cell line had lower levels of miR-16 expression than did the non–New Zealand Black B-cell line at all stages of the cell cycle, with $G_1$ phase cells having significantly higher levels of miR-16 (Fig. 3B). Transfection with 3 μg of miR-16 mimics resulted in an increase in the amount of

![Figure 2](image-url)
microRNA miR-16 in all cell cycle phases following transfection in the non–New Zealand Black and the New Zealand Black B-cell lines (Fig. 3C). Cell cycle analysis was done on each transfected fraction 24 hours posttransfection by staining with hypotonic propidium iodide. In all elutriated fractions studied, New Zealand Black had greatly elevated G1 arrest following miR-16 transfection when compared with the non–New Zealand Black B-cell line, with G1 accumulation in the New Zealand Black B-cell line being significantly greater in fraction 4 (S/G2; Fig. 3D).

**Restoration of miR-15a or miR-16 in the New Zealand Black–Derived Malignant B-1 Cell Line Correlates with a Decrease in Cyclin D1 Protein Levels**

We hypothesized that the G1 arrest observed in New Zealand Black cells upon transfection with miR-16 was a result of the targeting of a cell cycle regulator by miR-16. TargetScan, a microRNA target prediction program, listed cyclin D1 as a likely target for miR-15a and miR-16 in humans and mice. To determine if cyclin D1 is regulated by miR-15a or miR-16, we transfected the New Zealand Black and non–New Zealand Black B-cell lines with either microRNA mimic or the negative control mimic and evaluated cyclin D1 mRNA and protein levels at 24 hours posttransfection. PCR revealed no change in cyclin D1 mRNA levels upon miR-16 addition, indicating that cyclin D1 was being regulated by miR-16 posttranscriptionally (Fig. 4A). In addition, PCR analysis revealed cyclin D1a as the dominant isoform in the New Zealand Black B-cell line (as compared with cyclin D1b isoform). Cyclin D1a mRNA includes all three predicted miR-15a/16 target sequences in its 3' UTR.

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Cell cycle regulated expression of miR-16. New Zealand Black and non–New Zealand Black B-cell lines were separated into cell cycle phase fractions by centrifugal elutriation. **A**, representative flow cytometric profiles of elutriated B-cell lines analyzed for cell cycle phase based on DNA content. In the four panels depicting each elutriated fraction: leftmost gray peak, the G1 cells; rightmost gray peak, the G2 cells; and hatched area, the S phase (by computer modeling). Elutriated fractions: G1 (fraction 1), G1/S (fraction 2), S (fraction 3), and S/G2 (fraction 4). **B**, basal levels of miR-16 expression in elutriated cells separated into the various phases of the cell cycle. **C**, the levels of miR-16 expression 24 h posttransfection of the elutriated fractions with either negative control or miR-16 mimic. **D**, the elutriated fractions were transfected with miR-16 or negative control for 24 h. Change in the amount of cells in G1 phase is the mean percent change of miR-16 transfected cells compared with those transfected with negative control. The G1 accumulation seen in the New Zealand Black S/G2 cell fraction 4 is significantly higher than G1 accumulation in the other New Zealand Black fractions. *, P ≤ 0.02. Error bars, SEM for at least three independent experiments.
miR-15a, miR-16, or negative control. Flow cytometry was used 24 h posttransfection to evaluate the expression levels of GFP and mCherry.

Histograms are representative from four experiments. Percent decrease in mean fluorescence intensity of cyclin D1 protein levels 24 h after transfection with miR-16 as compared with negative control.

Cyclin D1 Is a Direct Target of miR-15a and miR-16

To confirm that miR-15a and miR-16 directly regulate cyclin D1, causing the observed decrease in protein levels, we cloned a 647-bp portion of the cyclin D1 3′ UTR containing miR-16 compared with the negative control (Table 1; Fig. 4B). Transfection with miR-15a also resulted in a decrease in cyclin D1 protein levels in the New Zealand Black B-cell line (Table 1).

Table 1. Effect of transfection of microRNAs on cyclin D1 levels

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment*</th>
<th>% Change cyclin D1</th>
</tr>
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<tbody>
<tr>
<td>NZB</td>
<td>miR-16</td>
<td>−24.61 ± 6.38</td>
</tr>
<tr>
<td></td>
<td>miR-15a</td>
<td>−28.54 ± 4.41</td>
</tr>
<tr>
<td>non-NZB</td>
<td>miR-16</td>
<td>−5.55 ± 5.40</td>
</tr>
<tr>
<td></td>
<td>miR-15a</td>
<td>−14.31 ± 4.31</td>
</tr>
</tbody>
</table>

NOTE: Mean fluorescence intensity as compared with negative control.

Abbreviation: NZB, New Zealand Black.

*Twenty-four hours posttransfection with 3 μg negative control, miR-16, or miR-15a.

based on TargetScan (data not shown). Flow cytometric analysis did not detect a significant change in cyclin D1 protein expression in the non–New Zealand Black B-cell line but did detect a substantial decrease in the mean fluorescence intensity of cyclin D1 protein in the New Zealand Black B-cell line transfected with miR-16 compared with the negative control (Table 1; Fig. 4B). Transfection with miR-15a also resulted in a decrease in cyclin D1 protein levels in the New Zealand Black B-cell line (Table 1).

Histograms are representative from four experiments. Percent decrease in mean fluorescence intensity of cyclin D1 protein levels 24 h after transfection with miR-16 as compared with negative control.

Cyclin D1 Is a Direct Target of miR-15a and miR-16

To confirm that miR-15a and miR-16 directly regulate cyclin D1, causing the observed decrease in protein levels, we cloned a 647-bp portion of the cyclin D1 3′ UTR containing all three predicted miR-15a/16 target sites downstream of the GFP expression cassette in a bidirectional lentiviral vector (Bd.LV.GFP.D1). The bidirectional lentiviral vector is an integrating vector that coordinately transcribes two transgenes, GFP and mCherry, as two distinct transcripts. Because the cyclin D1 3′ UTR is contained in the GFP cassette, expression of mCherry is unaffected by posttranscriptional regulation of GFP and thus serves as an internal control (42). We separately transduced our New Zealand Black–derived malignant B-1 cell line with the Bd.LV.GFP vector lacking the cyclin D1 3′ UTR sequence and with the Bd.LV.GFP.D1 vector containing the cyclin D1 3′ UTR. Each stably transduced line was then transfected with miR-15a and miR-16 mimics and a negative control. As expected, transfection with the microRNA mimics did not suppress expression of GFP from the Bd.LV.GFP vector, as indicated by the ratio of the mean fluorescence intensity between GFP and mCherry in cells treated with the microRNA or negative control mimic. Instead, flow cytometric analysis showed that transfection of the miR-15a and miR-16 mimics resulted in a significant decrease in GFP expression, but not mCherry expression, in cells with the Bd. LV.GFP.D1 vector (Fig. 4C).

We then used Amnis ImageStream analysis to look at individual New Zealand Black cells transduced with Bd.LV.GFP.D1. Similar to the results from flow cytometry, we found that GFP and mCherry were expressed at similar levels in cells treated with the negative control mimic.
However, treatment of these cells with either miR-15a or miR-16 decreased GFP expression, but not mCherry, with the overlay showing that a single cell produced mCherry but very little GFP (Fig. 4C). Together, these results show that miR-15a and miR-16 directly regulate cyclin D1 through target sites in the cyclin D1 3’ UTR.

**miR-16 Enhances the Ability of Nutlin and Genistein to Promote Apoptosis of Malignant B-1 Cells**

To determine whether restoring miR-16 in the New Zealand Black B-cell line would enhance drug sensitivity, the effect of exogenous miR-16 plus a chemotherapeutic agent, such as nutlin and genistein, was investigated. Nutlin, a small-molecule antagonist of MDM2, has been shown to activate p53 and induce apoptosis in cancer cells, including B-cell CLL (34). Genistein, an isoflavone derived from soybeans that also down-regulates cyclin D1, has also been shown to inhibit the growth of malignant cells while preserving normal cells (35). A dose-response curve was generated for nutlin and genistein, treating the New Zealand Black B-cell line with varying micromolar concentrations (Supplementary Fig. S1A and B). Suboptimal doses, at which the percentage of apoptotic cells were 10% to 15% of the maximum apoptotic level reached, were used to execute further experimentation in this report. We treated our New Zealand Black–derived malignant B-1 cell line and a non–New Zealand Black B-cell line with either 5 μmol/L of nutlin or 10 μmol/L of genistein alone and in conjunction with 100 nmol/L exogenous miR-16. Treating the New Zealand Black B-cell line with miR-16 and nutlin increased the percentage of cells in sub-G1 (Fig. 5A), providing a synergistic effect, significantly enhancing apoptosis at 24 and 48 hours in the New Zealand Black B-cell line, but not in the non–New Zealand Black cell line (Fig. 5B). The addition of miR-16 significantly enhanced genistein-induced apoptosis.

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**Figure 5.** miR-16 enhances the apoptotic effects of nutlin and genistein on the New Zealand Black malignant B-1 cell line. The New Zealand Black B-cell line was treated with either 5 μmol/L nutlin or 10 μmol/L genistein alone or in conjunction with miR-16 or mimic negative control. Cells were stained with propidium iodide 24 and 48 h posttreatment to analyze DNA content and detect apoptosis levels. A, 24-h representative analysis of DNA content in untreated or treated (with nutlin and/or miR-16) New Zealand Black B-cell line. In each of the panels, the percentage of apoptotic cells is above the peak representing sub-G1 (shaded peaks are computer modeled). B, Δs of the percentage of cells undergoing apoptosis in response to nutlin treatment, transfection with miR-16 or negative control, and nutlin + miR-16 or negative control in non–New Zealand Black and New Zealand Black B-cell lines 24 and 48 h posttreatment. Black columns, difference in percent apoptosis between nutlin-treated and untreated cells. White, difference between cells transfected with miR-16 and those with negative control. Gray, difference between nutlin + miR-16 and nutlin + negative control. C, Δs of the percentage of apoptosis in response to genistein treatment, transfection with miR-16 or negative control, and genistein + miR-16 or genistein + negative control in non–New Zealand Black and New Zealand Black B-cell lines 24 and 48 h posttreatment. Black columns, difference in percent apoptosis between genistein-treated and untreated cells. White, difference between miR-16 and negative control. Gray, difference between genistein + miR-16 and genistein + negative control. * indicates statistical significance (P < 0.05) in apoptotic increase upon addition of miR-16 to nutlin-treated and genistein-treated New Zealand Black B-cell lines; #, statistical significance (P < 0.02) in apoptotic increase between the New Zealand Black and non–New Zealand Black B-cell lines. Dashed line, predicted value if effects of nutlin (or genistein) and miR-16 were additive. The synergistic effect seen upon addition of miR-16 to genistein at 48 h was statistically significant (when compared with the predicted additive value, t, P < 0.05). Error bars, SEM for at least three independent experiments.
in the New Zealand Black B-cell line at 24 and 48 hours, while having little to no effect in the non–New Zealand Black B-cell line, which had normal levels of miR-16 (Fig. 5C). At 48 hours, the combination treatment of miR-16 and genistein resulted in a significant synergistic induction of apoptosis (Fig. 5C).

Discussion

In this report, we found exogenous restoration of miR-16 to have cell cycle effects in the New Zealand Black B-1 cell line but little to no effect in non–New Zealand Black B-cell lines expressing normal levels of miR-16. The addition of miR-16 to the New Zealand Black B-cell line resulted in a G1 arrest at all stages of the cell cycle and a decrease in cyclin D1 protein levels, as well as a sensitization to the apoptotic effects of nutlin and genistein.

The New Zealand Black B-1 cell line has decreased expression of miR-16 relative to the non–New Zealand Black B-cell line expression at all stages of the cell cycle. Because the alterations in the primary mir-15a/16-1 loci were linked to the development of CLL in the New Zealand Black murine model (14), we investigated the role of miR-16 in cell cycle regulation and whether increasing miR-16 expression would alter the cell cycle and thereby malignant B-1 cell growth. In non–New Zealand Black and New Zealand Black B-cell lines, increasing miR-16 expression resulted in G1 accumulation; however, the effect of miR-16 was much more pronounced in the New Zealand Black B-1 cell line. In addition, the cells in the late S and G2/M (S/G2) phase of the cell cycle were most sensitive to the effects of miR-16 transfection, showing the most G1 accumulation.

One of the reported targets of miR-16 in human and mouse is cyclin D1 (32, 33). In the present report, reduced levels of miR-16 in the New Zealand Black correlates with the overexpression of the target gene CCND1 (cyclin D1). Among leukemias and lymphomas, elevated levels of cyclin D1 have primarily been associated with mantle cell lymphoma (29); however, reports have shown some CLL patients with high levels of cyclin D1 (30). Cyclin D1 and D3 levels have been reported to be higher in leukemic cells than in normal circulating B cells (43). In addition, increased levels of cyclin D1 were reported in CD5+ zap70+ CLL cells as compared with CD5+ zap70− CLL cells (44), as well as in a subset of patients with clinically aggressive CLL (45). Our New Zealand Black–derived malignant B-1 cell line, LNC, mimics late-stage aggressive CLL (27). Deletions in the 3′ UTR of cyclin D1 have been reported in CLL and are associated with overexpression of cyclin D1 (46, 47).

The New Zealand Black B cells have elevated cyclin D1 levels, which are similar to the elevated levels reported in some cases of human CLL. The previously reported mean fluorescence intensity ratio of cyclin D1 relative to isotype in patients with CLL had a range of 5.6 to 8.3 mean fluorescence intensity ratio (30), which is similar to that herein reported for untransfected New Zealand Black malignant B-1 cells (6.2 mean fluorescence intensity ratio). The mean fluorescence intensity ratio of non-CLL normal control individuals (4.8 mean fluorescence intensity ratio) was similar to the cyclin D1 levels following restoration of miR-16 in the New Zealand Black B-1 cells (4.9 mean fluorescence intensity ratio). Thus, exogenous miR-16 restored the level of cyclin D1 in the New Zealand Black B-cell line to a level similar to normal cells.

A selective effect of exogenous miR-16 on malignant cells, while sparing surrounding normal cells, has also been observed in nonlymphoid cancers resulting in G1 arrest and decreased cyclin D1 (24, 26, 32, 33). We propose that decreased expression of cyclin D1, resulting from elevated miR-16 levels following transfection, played a significant role in the New Zealand Black B-cell line accumulation in G1. miR-16 was also shown to act synergistically with drugs nutlin and genistein to selectively increase the percentage of cells undergoing apoptosis in the New Zealand Black malignant B-cell line but not in the non–New Zealand Black B-cell line.

Our data support the involvement of microRNAs in cancer (48) and show miR-16 as a key regulator of cell cycle progression, G1/S transition in particular. miR-16 may be used in a therapeutic approach to target and down-regulate oncogenic proteins overexpressed in cancers, such as cyclin D1. Restoration of miR-16 levels may also be used to increase apoptosis induced by other agents, increasing drug sensitivity in malignant cells deficient in miR-16.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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Molecular Cancer Therapeutics

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